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A review of the available literature on the known microbial metabolites of the explosive TNT has been carried out. Of the nine metabolites that have been identified and characterized in the reductive biotransformation pathway, only five acute toxicity studies have been reported on 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. The results indicate that they are not acutely toxic in rats and mice. The Ames Salmonella bioassay was the only other study reported by three different groups of investigators. Only seven of the nine metabolites were tested: they were 2-hydroxylamino-4,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,4,-diamino-6-nitrotoluene, 4,4',6,6'-tetranitro-2,2'-azoxytoluene and 2,2',6,6'-tetranitro-4,4'-azoxytoluene. Anomalous results were found and reported by all workers using this assay. For the ultimate determination of NOEL/NOAEL values to be made, numerous further studies need to be carried out, and recommendations for such studies have been made.

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Project Managers Signature — Date Oct. 22, 1993

TABLE OF CONTENTS

List of Figures

List of Tables

TOXICITY OF MICROBIAL METABOLITES OF 2,4,6-TRINITROTOLUENE (TNT)

The documentation of the biological degradation of nitroaromatic explosives, TNT in particular, that results when these compounds are disposed of in soil and water, is extensive. The distribution, identification and biological fate of many of the degraded compounds or pollutants, particularly in soil, have been studied mainly at the U.S. Army Natick R&D Center, Natick, and also at the U.S. Army Biomedical R&D Laboratory, Fort Detrick. The microbiological transformations of TNT by reductive reactions have recently been reviewed by Walsh (1990), Rosenblatt, *et al.* (1991) and Walker and Kaplan (1992) and thus provide the background for this report.

Currently, there are many studies being carried out on the degradation of TNT using composting processes (Tan, *et al.*, 1992, Griest, *et al.*, 1993). The results from these studies indicate that the reductive biodegradation pathway of TNT by compost micro-organisms is essentially the same as that reported for the numerous soil and fresh water micro-organisms that have been studied (Rosenblatt, *et al.*, 1991). Apart from the photochemical transformations of TNT, there is as yet, no evidence for other important chemical or biological transformation processes such as hydrolysis or oxidation under environmental conditions (Rosenblatt, *et al.*, 1991).

MICROBIAL METABOLITES OF TNT

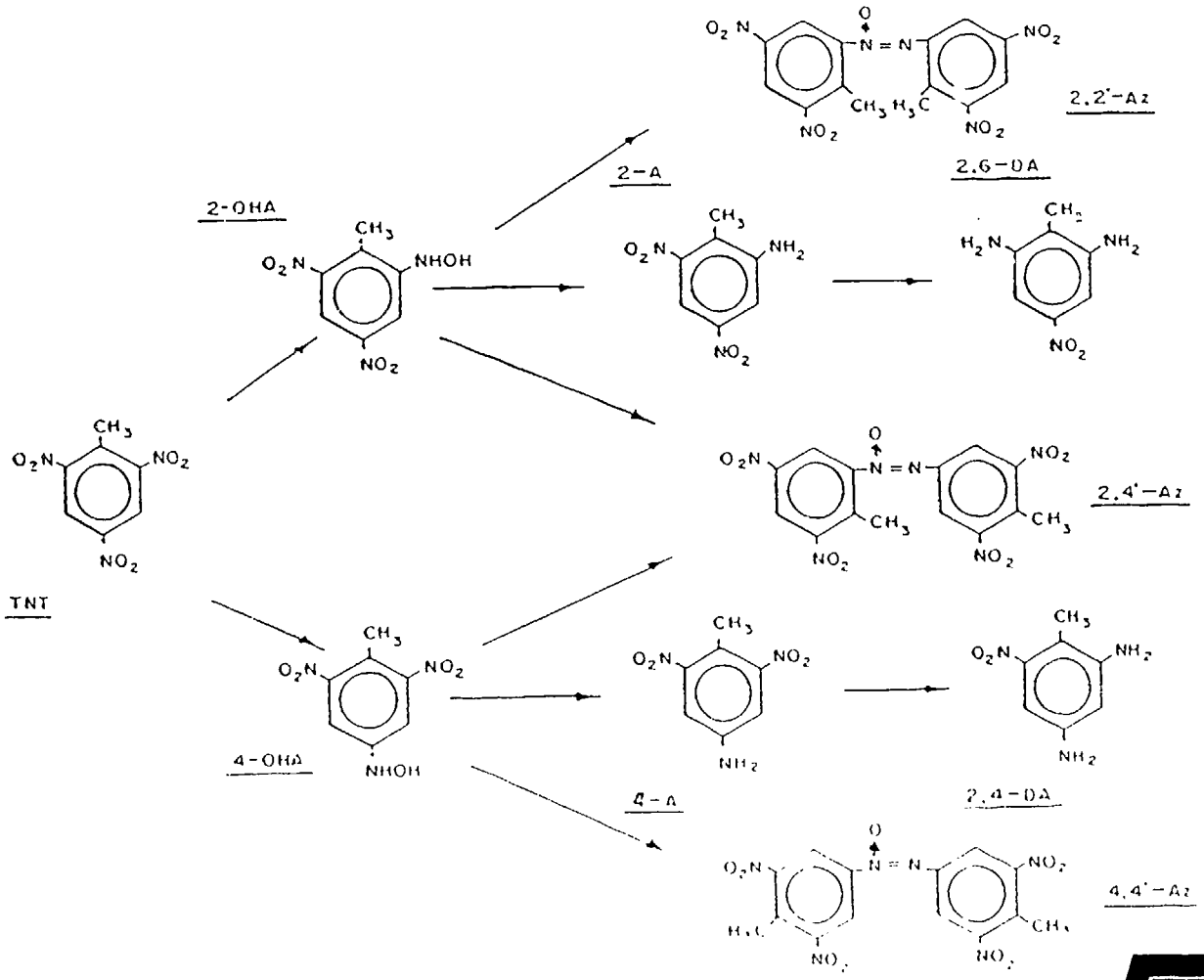
There have been nine microbial metabolites of TNT identified and characterized in both field and laboratory studies. These compounds are listed in Table 1 along with their acronyms and Chemical Abstract Service (CAS) registry numbers. The reductive biotransformation pathway for TNT is shown in Figure 1 (Walsh, 1990). Successive reduction of the nitro groups to amino groups is thought to proceed through the hydroxylamine intermediates (not isolated usually), of which the two that have been identified (2-OHA, 4-OHA) can then couple to form the identified tetranitroazoxytoluenes (Table 1). The compounds predominating at any one time will depend on the physico-chemical nature of the environment, the species of micro-organisms involved, and the conditions under which each nitro group reduction takes place.

TOXICITY OF TNT METABOLITES

An extensive search of the established toxicological information bases was made for toxicity data on all the compounds listed in Table 1. The only toxicities found were Ames *Salmonella* assays on seven of the nine metabolites listed in Table 1 (exceptions were 2,6-DA and 2,4'-Az) by Won, *et al.* (1976), Spanggord, *et al.* (1982) and Tan, *et al.* (1992), and five acute studies on two of the mono-amino dinitrotoluenes (2-A and 4-A) by Ellis, *et al.*, (1978, 1980).

Table 1. Microbial Metabolites of TNT

Metabolite	Acronym	C.A.S. No.
2-Hydroxylamino-4,6-dinitrotoluene	2-OHA	59283-76-0
2-Amino-4,6-dinitrotoluene	2-A	35572-78-2
2,6-Diamino-4-nitrotoluene	2,6-DA	59229-75-3
4,4',6,6'-Tetranitro-2,2'-azoxytoluene	2,2'-Az	35212-01-2
4-Hydroxylamino-2,6-dinitrotoluene	4-OHA	59283-75-9
4-Amino-2,6-dinitrotoluene	4-A	19406-51-0
2,4-Diamino-6-nitrotoluene	2,4-DA	6629-29-4
2,2',6,6'-Tetranitro-4,4'-azoxytoluene	4,4'-Az	51857-25-1
4,2',6,6'-Tetranitro-6,4'-azoxytoluene	2,4'-Az	51856-71-4



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Won, *et al.* (1976) tested seven of the metabolites using the histidine-requiring strains of *Salmonella typhimurium*, both with and without S9 activation as first described by Ames. Revertant colony counts of the tester strains used (not identified by number) were essentially similar to those of the controls, indicating that all the compounds were non-reactive and non-mutagenic.

Spanggord, *et al.* (1982) at the Stanford Research Institute, Palo Alto, reported mixed results for both compounds 2-A and 4-A in the *Salmonella* assay using five strains (TA98, TA100, TA1535, TA1537, TA1538) of S. typhimurium, with and without S9 activation, and concentrations of 10 - 5000 μg/plate. Metabolite 2-A was found to be positive both with and without activation in TA98, TA100, TA1537, TA1538. In TA1535 it was positive with activation and negative without activation and was negative also in the TA100 NR3 strain. Metabolite 4-A was found to be positive, both with and without activation in TA98 and TA100. It was negative both with and without activation in TA1537 and a TA100 NR3 strain. In TA1535 it was positive with activation, but negative without activation. In TA1538 it was positive without activation but negative with activation. Tan, *et al.* (1992) also used the Ames assay using two strains of *Salmonella* (TA98, TA100) both with and without S9 activation to test metabolites 2-A, 2,6-DA, 4-A, 2,4-DA. The results were reported to be weakly variable in mutagenic activity. In summary, there are highly conflicting results reported in the three studies, especially for the metabolites 2-A and 4-A. The Ames assay should be repeated on all nine compounds.

The results of the five acute studies on metabolites 2-A and 4-A are set out in Table 2. Both compounds were found to be essentially non-toxic to rats and mice in oral LD50 studies, and apart from a relatively mild irritant effect of compound 2-A on rabbit skin, they were both shown to be non-irritant to the eyes of rabbits and non-sensitive to the skin of guinea pigs. This work was carried out at the Midwest Research Institute, Kansas City by Ellis, *et al.* (1978, 1980).

Table 2. Acute Toxicities of Two TNT Metabolites

Metabolite	LD50 Mouse*	LD50 Rat*	Rabbit Eye Irritation	Rabbit Skin Irritation	Dermal Sensitivity
2-A	1722±154(M) 1522±71 (F)	2240±85(M) 1394±191(F)	Non-irritant	Mild irritant	None
4-A	1342±107(M) 1495±90 (F)	1360±53(M) 959±76(F)	Non-irritant	Non-irritant	None

* mg/kg. ± S.D. (M = Male, F = Female)

DATA GAPS AND RECOMMENDATIONS FOR FURTHER RESEARCH

There is an obvious dearth of toxicity data on all of the nine identified metabolites of TNT. It is recommended that further toxicity studies be undertaken and carried out in four phases (Table 3). If positive results are determined from both the phase I and II studies then proceed to phase III and if necessary to phase IV studies. It would appear that only seven of the nine metabolites need to be studied and evaluated in each phase, and especially since the two metabolites, 2-OHA and 4-OHA, are very rapidly converted to their corresponding amino and azo-derivatives, they could be excluded. In phase I, only three appropriate assays should be selected for study, and in phase II it would appear unnecessary to repeat the acute studies on metabolities 2-A and 4-A as reported by Ellis, *et al.* (1978, 1980). From the available toxicity data on these compounds and the obvious lack of subacute and chronic studies, it is not feasible to estimate any NOEL or NOAEL values for any of these metabolites of TNT.

Table 3. Recommended Toxicity Studies on the TNT Metabolites (Listed in Order of Importance)

Phase I:	1. Ames *Salmonella* assay 2. Mouse lymphoma assay 3. CHO mutation assay (in vitro) 4. SCE mutation assay (in vivo) 5. DNA synthesis assay 6. Micronucleus assay	**Phase II:**	1. Acute LD50-rats, mice 2. Eye irritation - rabbits 3. Skin irritation - rabbits 4. Dermal sensitivity - guinea pigs
Phase III:	1. 90-Day subchronic - rats 2. Teratology - rats, rabbits 3. Dominant lethal - rats 4. Reproductive assessment by continuous breeding - rats 5. Toxicokinetics and metabolic fate studies - rats or mice	**Phase IV:**	Chronic lifetime - rats or Carcinogenicity - rats or mice

SUMMARY

A review of the available literature on the known microbial metabolites of the explosive TNT has been carried out. Of the nine metabolites that have been identified and characterized in the reductive biotransformation pathway, only five acute toxicity studies have been reported on 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. The results indicate that they are

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REFERENCES

Ellis, H.V. III, Hodgson, J.R., Hwang, S.W., Halpap, L.M., Helton, D.L., Andersen, B.S., VanGoethem, D.L. and Lee, C.C. Mammalian Toxicity of Munitions Compounds Phase I: Acute Oral Toxicity, Primary Skin and Eye Irritation, Dermal Sensitization, Disposition and Metabolism, and Ames Tests of Additional Compounds. Progress Report No. 6, prepared by Midwest Research Institute, Kansas City, for U.S. Army Medical Research and Development Command under Contract No. DAMD17-74-C-4073 (AD A069333) (1978).

Ellis, H.V. III, Hong, C.B. and Lee, C.C. Mammalian Toxicity of Munitions Compounds: Summary of Toxicity of Nitrotoluenes. Progress Report No. 11, prepared by Midwest Research Institute, Kansas City, for U.S. Army Medical Research and Development Command under Contract No. DAMD17-74-C-4073 (AD A080146) (1980).

Gordon, L. and Hartley, W.R. 2,4,6-Trinitrotoluene (TNT). In *Drinking Water Health Advisory: Munitions*, pp. 327-398. Ed. by Roberts, W.C. and Hartley, W.R. and publ. by Lewis Publishers, Boca Raton (1992).

Griest, W.H., Stewart, A.J., Tyndall, R.L., Caton, J.E., Ho, C.H., Ironside, K.S., Caldwell, W.M. and Tan, E. Chemical and Toxicological Testing of Composted Explosives-Contaminated Soil. *Environ. Toxicol. Chem.* **12:** 1105-1116 (1993).

Rosenblatt, D.H., Burrows, E.P., Mitchell, W.R. and Parmer, D.L. Organic Explosives and Related Compounds. In *The Handbook of Environ. Chem.*, **3, Pt.G:** 195-234. Ed. by Hutzinger, O. and publ. by Springer-Verlag, Berlin (1991).

Spanggord, R.J., Mortelmans, K.E., Griffin, A.F. and Simmon, V.F. Mutagenicity in *Salmonella typhimurium* and Structure-Activity Relationships of Wastewater Components Emanating From the Manufacture of Trinitrotoluene. *Environ. Mutagen.* **4:** 163-179 (1982).

Tan, E.L., Ho, C.H., Griest, W.H. and Tyndall, R.L. Mutagenicity of Trinitrotoluene and its Metabolites Formed During Composting. *J. Toxicol. Environ. Hlth.* **36:** 165-175 (1992).

Walker, J.E. and Kaplan, D.L. Biological Degradation of Explosives and Chemical Agents. *Biodegradation* **3:** 369-385 (1992).

Walsh, M.E. Environmental Transformation Products of Nitroaromatics and Nitramines. Literature Review and Recommendations for Analytical Development. Special Report 90-2 prepared by Cold Regions Research & Engineering Laboratory, U.S. Army Corps of Engineers, Hanover, for U.S. Army Toxic and Hazardous Materials Agency (CETHA-TE-CR-89205) (1990).

Won, W.D., DiSalvo, L.H. and Ng, J. Toxicity and Mutagenicity of 2,4,6-Trinitrotoluene and its Microbial Metabolites. *Appl. Environ. Microbiol.* **31:** 576-580 (1976).

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